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(54) Title: HEAT STABLE MUTANTS OF STARCH BIOSYNTHESIS ENZYMES

(57) Abstract

The subject invention pertains to novel mutant polynucleotide molecules that encode enzymes that have increased heat stability. These polynucleotides, when expressed in plants, result in increased yield in plants grown under conditions of heat stress. The polynucleotide molecules of the subject invention encode maize endosperm ADP glucose pyrophosphorylase (AGP) and soluble starch synthase (SSS) enzyme activities. Plants and plant tissue bred to contain, or transformed with, the mutant polynucleotides, and expressing the polypeptides encoded by the polynucleotides, are also contemplated by the present invention. The subject invention also concerns methods for isolating polynucleotides and polypeptides contemplated within the scope of the invention. Methods for increasing yield in plants grown under conditions of best stress are also provided.

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DESCRIPTION

HEAT STABLE MUTANTS OF STARCH BIOSYNTHESIS ENZYMES

This invention was made with government support under National Science Foundation grant number 9316887. The government has certain rights in the invention.

Cross-Reference to Related Applications

This application claims the benefit of U.S. Provisional Application No. 60/031,045, filed November 18, 1996.

Background of the Invention

The sessile nature of plant life generates a constant exposure to environmental factors that exert positive and negative effects on its growth and development. One of the major impediments facing modern agriculture is adverse environmental conditions. One important factor which causes significant crop loss is heat stress. Temperature stress greatly reduces grain yield in many cereal crops such as maize, wheat, and barley. Yield decreases due to heat stress range from 7 to 35% in the cereals of world-wide importance.

A number of studies have identified likely physiological consequences of heat stress. Early work by Hunter *et al.* (Hunter, R. B., Tollenaar, M., and Breuer, C. M. [1977] *Can. J. Plant Sci.* 57:1127-1133) using growth chamber conditions showed that temperature decreased the duration of grain filling in maize. Similar results in which the duration of grain filling was adversely altered by increased temperatures were identified by Tollenaar and Bruulsema (Tollenaar, M. and Bruulsema, T. W. [1988] *Can. J. Plant Sci.* 68:935-940). Badu-Apraku *et al.* (Badu-Apraku, B., Hunter, R. B., and Tollenaar, M. [1983] *Can. J. Plant. Sci.* 63:357-363) measured a marked reduction in the yield of maize plants grown under the day/night temperature regime of 35/15°C compared to growth in a 25/15°C temperature regime. Reduced yields due to increased temperatures is also supported by historical as well as climatological studies (Thompson, L. M. [1986]

Agron. J. 78:649-653; Thompson, L. M. [1975] *Science* 188:535-541; Chang, J. [1981] *Agricul. Metero.* 24:253-262; and Conroy, J. P., Seneweera, S., Basra, A. S., Rogers, G., and Nissen-Wooller, B. [1994] *Aust. J. Plant Physiol.* 21:741-758).

That the physiological processes of the developing seed are adversely affected by heat stress is evident from studies using an *in vitro* kernel culture system (Jones, R.J., Gengenbach, B.G., and Cardwell, V.B. [1981] *Crop Science* 21:761-766; Jones, R.J., Ouattar, S., and Crookston, R.K. [1984] *Crop Science* 24:133-137; and Cheikh, N., and Jones, R.J. [995] *Physiol. Plant.* 95:59-66). Maize kernels cultured at the above-optimum temperature of 35°C exhibited a dramatic reduction in weight.

Work with wheat identified the loss of soluble starch synthase (SSS) activity as a hallmark of the wheat endosperm's response to heat stress (Hawker, J. S. and Jenner, C. F. [1993] *Aust. J. Plant Physiol.* 20:197-209; Denyer, K., Hylton, C. M., and Smith, A. M. [1994] *Aust. J. Plant Physiol.* 21:783-789; Jenner, C. F. [1994] *Aust. J. Plant Physiol.* 21:791-806). Additional studies with SSS of wheat endosperm show that it is heat labile (Rijven, A.H.G.C. [1986] *Plant Physiol.* 81:448-453; Keeling, P.L., Bacon, P.J., Holt, D.C. [1993] *Planta.* 191:342-348; Jenner, C. F., Denyer, K., and Guerin, J. [1995] *Aust. J. Plant Physiol.* 22:703-709).

The roles of SSS and ADP glucose pyrophosphorylase (AGP) under heat stress conditions in maize is less clear. (AGP) catalyzes the conversion of ATP and α-glucose-1-phosphate to ADP-glucose and pyrophosphate. ADP-glucose is used as a glycosyl donor in starch biosynthesis by plants and in glycogen biosynthesis by bacteria. The importance of ADP-glucose pyrophosphorylase as a key enzyme in the regulation of starch biosynthesis was noted in the study of starch deficient mutants of maize (*Zea mays*) endosperm (Tsai, C.Y., and Nelson, Jr., O.E. [1966] *Science* 151:341-343; Dickinson, D.B., J. Preiss [1969] *Plant Physiol.* 44:1058-1062).

Ou-Lee and Setter (Ou-Lee, T. and Setter, T.L. [1985] *Plant Physiol.* 79:852-855) examined the effects of temperature on the apical or tip regions of maize ears. With elevated temperatures, AGP activity was lower in apical kernels when compared to basal kernels during the time of intense starch deposition. In contrast, in kernels developed at normal temperatures, AGP activity was similar in apical and basal kernels during this period. However, starch synthase activity during this period was not differentially

affected in apical and basal kernels. Further, heat-treated apical kernels exhibited an increase in starch synthase activity over control. This was not observed with AGP activity. Singletary *et al.* (Singletary, G.W., Banisadr, R., and Keeling, P.L. [1993] *Plant Physiol.* 102: 6 (suppl).; Singletary, G.W., Banisadra, R., Keeling, P.L. [1994] *Aust. J. Plant Physiol.* 21:829-841) using an *in vitro* culture system quantified the effect of various temperatures during the grain fill period. Seed weight decreased steadily as temperature increased from 22-36°C. A role for AGP in yield loss is also supported by work from Duke and Doehlert (Duke, E.R. and Doehlert, D.C. [1996] *Environ. Exp. Botany.* 36:199-208).

Work by Keeling *et al.* (1994, *supra*) quantified SSS activity in maize and wheat using Q_{10} analysis, and showed that SSS is an important control point in the flux of carbon into starch.

In vitro biochemical studies with AGP and SSS clearly show that both enzymes are heat labile. Maize endosperm AGP loses 96% of its activity when heated at 57°C for five minutes (Hannah, L.C., Tuschall, D.M., and Mans, R.J. [1980] *Genetics* 95:961-970). This is in contrast to potato AGP which is fully stable at 70°C (Sowokinos, J.R. and Preiss, J. [1982] *Plant Physiol.* 69:1459-1466; Okita, T.W., Nakata, P.A., Anderson, J.M., Sowokinos, J., Morell, J., and Preiss, J. [1990] *Plant Physiol.* 93:785-90). Heat inactivation studies with SSS showed that it is also labile at higher temperatures, and kinetic studies determined that the K*m* value for amylopectin rose exponentially when temperature increased from 25-45°C (Jenner *et al.*, 1995, *supra*).

Biochemical and genetic evidence has identified AGP as a key enzyme in starch biosynthesis in higher plants and glycogen biosynthesis in *E. coli* (Preiss, J. and Romeo, T. [1994] *Progress in Nuc. Acid Res. and Mol Biol.* 47:299-329; Preiss, J. and Sivak, M. [1996] "Starch synthesis in sinks and sources," In *Photoassimilate distribution in plants and crops: source-sink relationships*. Zamski, E., ed., Marcil Dekker Inc. pp. 139-168). AGP catalyzes what is viewed as the initial step in the starch biosynthetic pathway with the product of the reaction being the activated glucosyl donor, ADPglucose. This is utilized by starch synthase for extension of the polysaccharide polymer (reviewed in Hannah, L. Curtis [1996] "Starch synthesis in the maize endosperm," In: *Advances in Cellular and Molecular Biology of Plants*, Vol. 4. B. A. Larkins and I. K. Vasil (eds.).

Cellular and Molecular Biology of Plant Seed Development. Kluwer Academic Publishers, Dordrecht, The Netherlands, (in press)).

Initial studies with potato AGP showed that expression in *E. coli* yielded an enzyme with allosteric and kinetic properties very similar to the native tuber enzyme (Iglesias, A., Barry, G.F., Meyer, C., Bloksberg, L., Nakata, P., Greene, T., Laughlin, M.J., Okita, T.W., Kishore, G.M., and Preiss, J. [1993] *J. Biol Chem.* 268:1081-86; Ballicora, M.A., Laughlin, M.J., Fu, Y., Okita, T.W., Barry, G.F., and Preiss, J. [1995] *Plant Physiol.* 109:245-251). Greene *et al.* (Greene, T.W., Chantler, S.E., Kahn, M.L., Barry, G.F., Preiss, J., and Okita, T.W. [1996] *Proc. Natl. Acad. Sci.* 93:1509-1513; Greene, T.W., Woodbury, R.L., and Okita, T.W. [1996b] *Plant Physiol.* (in press)) showed the usefulness of the bacterial expression system in their structure-function studies with the potato AGP. Multiple mutations important in mapping allosteric and substrate binding sites were identified (Okita, T.W., Greene, T.W., Laughlin, M.J., Salamone, P., Woodbury, R., Choi, S., Ito, H., Kavakli, H., and Stephens, K. [1996] "Engineering Plant Starches by the Generation of Modified Plant Biosynthetic Enzymes," In *Engineering Crops for Industrial End Uses*, Shewry, P.R., Napier, J.A., and Davis, P., eds., Portland Press Ltd., London. (in press)).

AGP enzymes have been isolated from both bacteria and plants. Bacterial AGP consists of a homotetramer, while plant AGP from photosynthetic and non-photosynthetic tissues is a heterotetramer composed of two different subunits. The plant enzyme is encoded by two different genes, with one subunit being larger than the other. This feature has been noted in a number of plants. The AGP subunits in spinach leaf have molecular weights of 54 kDa and 51 kDa, as estimated by SDS-PAGE. Both subunits are immunoreactive with antibody raised against purified AGP from spinach leaves (Copeland, L., J. Preiss (1981) *Plant Physiol.* 68:996-1001; Morell, M., M. Bloon, V. Knowles, J. Preiss [1988] *J. Bio. Chem.* 263:633). Immunological analysis using antiserum prepared against the small and large subunits of spinach leaf showed that potato tuber AGP is also encoded by two genes (Okita *et al.*, 1990, *supra*). The cDNA clones of the two subunits of potato tuber (50 and 51 kDa) have also been isolated and sequenced (Muller-Rober, B.T., J. Kossmann, L.C. Hannah, L. Willmitzer, U. Sonnewald [1990] *Mol. Gen. Genet.* 224:136-146; Nakata, P.A., T.W. Greene, J.M. Anderson, B.J.

Smith-White, T.W. Okita, J. Preiss [1991] *Plant Mol. Biol.* 17:1089-1093). The large subunit of potato tuber AGP is heat stable (Nakata *et al.* [1991], *supra*).

As Hannah and Nelson (Hannah, L.C., O.E. Nelson (1975) *Plant Physiol.* 55:297-302.; Hannah, L.C., and Nelson, Jr., O.E. [1976] *Biochem. Genet.* 14:547-560) postulated, both *Shrunken-2* (*Sh2*) (Bhave, M.R., S. Lawrence, C. Barton, L.C. Hannah [1990] *Plant Cell* 2:581-588) and *Brittle-2* (*Bt2*) (Bae, J.M., M. Giroux, L.C. Hannah [1990] *Maydica* 35:317-322) are structural genes of maize endosperm ADP-glucose pyrophosphorylase. *Sh2* and *Bt2* encode the large subunit and small subunit of the enzyme, respectively. From cDNA sequencing, *Sh2* and *Bt2* proteins have predicted molecular weight of 57,179 Da (Shaw, J.R., L.C. Hannah [1992] *Plant Physiol.* 98:1214-1216) and 52,224 Da, respectively. The endosperm is the site of most starch deposition during kernel development in maize. *Sh2* and *bt2* maize endosperm mutants have greatly reduced starch levels corresponding to deficient levels of AGP activity. Mutations of either gene have been shown to reduce AGP activity by about 95% (Tsai and Nelson, 1966, *supra*; Dickinson and Preiss, 1969, *supra*). Furthermore, it has been observed that enzymatic activities increase with the dosage of functional wild type *Sh2* and *Bt2* alleles, whereas mutant enzymes have altered kinetic properties. AGP is the rate limiting step in starch biosynthesis in plants. Stark *et al.* placed a mutant form of *E. coli* AGP in potato tuber and obtained a 35% increase in starch content (Stark *et al.* [1992] *Science* 258:287).

The cloning and characterization of the genes encoding the AGP enzyme subunits have been reported for various plants. These include *Sh2* cDNA (Bhave *et al.*, 1990, *supra*), *Sh2* genomic DNA (Shaw and Hannah, 1992, *supra*), and *Bt2* cDNA (Bae *et al.*, 1990, *supra*) from maize; small subunit cDNA (Anderson, J.M., J. Hnilo, R. Larson, T.W. Okita, M. Morell, J. Preiss [1989] *J. Biol. Chem.* 264:12238-12242) and genomic DNA (Anderson, J.M., R. Larson, D. Landencia, W.T. Kim, D. Morrow, T.W. Okita, J. Preiss [1991] *Gene* 97:199-205) from rice; and small and large subunit cDNAs from spinach leaf (Morell *et al.*, 1988, *supra*) and potato tuber (Muller-Rober *et al.*, 1990, *supra*; Nakata, P.A., Greene, T.W., Anderson, J.W., Smith-White, B.J., Okita, T.W., and Preiss, J. [1991] *Plant Mol. Biol.* 17:1089-1093). In addition, cDNA clones have been isolated from wheat endosperm and leaf tissue (Olive, M.R., R.J. Ellis, W.W. Schuch

[1989] *Plant Physiol. Mol. Biol.* 12:525-538) and *Arabidopsis thaliana* leaf (Lin, T., Caspar, T., Sommerville, C.R., and Preiss, J. [1988] *Plant Physiol.* 88:1175-1181).

AGP functions as an allosteric enzyme in all tissues and organisms investigated to date. The allosteric properties of AGP were first shown to be important in *E. coli.* A glycogen-overproducing *E. coli* mutant was isolated and the mutation mapped to the structural gene for AGP, designated as *gly*C. The mutant *E. coli*, known as *gly*C-16, was shown to be more sensitive to the activator, fructose 1,6 bisphosphate, and less sensitive to the inhibitor, cAMP (Preiss, J. [1984] *Ann. Rev. Microbiol.* 419-458). Although plant AGP's are also allosteric, they respond to different effector molecules than bacterial AGP's. In plants, 3-phosphoglyceric acid (3-PGA) functions as an activator while phosphate (PO_4) serves as an inhibitor (Dickinson and Preiss, 1969, *supra*).

Using an *in vivo* mutagenesis system created by the *Ac*-mediated excision of a *Ds* transposable element fortuitously located close to a known activator binding site, Giroux *et al.* (Giroux, M.J., Shaw, J., Barry, G., Cobb, G.B., Greene, T., Okita, T.W., and Hannah, L. C. [1996] *Proc. Natl. Acad. Sci.* 93:5824-5829) were able to generate site-specific mutants in a functionally important region of maize endosperm AGP. One mutant, *Rev 6,* contained a tyrosine-serine insert and conditioned a 11-18% increase in seed weight.

Brief Summary of the Invention

The subject invention pertains to materials and methods useful for improving crop yields in plants, such as those plants that produce cereal crops. In one embodiment, the subject invention provides heat stable AGP enzymes and nucleotide sequences which encode these enzymes. In a preferred embodiment, the heat stable enzymes can be used to provide plants having greater tolerance to higher temperatures, thus enhancing the crop yields from these plants. In a particularly preferred embodiment, the improved plant is a cereal. Cereals to which this invention applies include, for example, maize, wheat, rice, and barley.

Brief Description of the Drawings

Figure 1. Heat stable maize endosperm AGP large subunit mutants. Percentage of AGP activity remaining after five minutes of heat treatment at 60°C is shown.

Figure 2. Primary sequence alignment of region surrounding HS 33 mutation with large subunits of maize, wheat, barley, and potato. Conserved regions are boxed.

Figure 3. Primary sequence alignment of region surrounding HS 40 mutation with large subunits of maize, wheat, barley, and potato. Conserved regions are boxed. Bolded aspartic acid residue corresponds to D413A allosteric mutant of potato LS (Greene, T.W., Woodbury, R.L., and Okita, T.W. [1996] *Plant Physiol.* (in press)). Spinach leaf AGP sequence is the activator site 2 peptide identified in 3-PGA analogue studies (Ball, K. and Preiss, J. [1994] *J. Biol. Chem.* 269:24706-24711). The labeled lysine residue is bolded.

Detailed Disclosure of the Invention

The subject invention concerns novel mutant polynucleotide molecules, and the polypeptides encoded thereby, that confer increased yield in plants grown under conditions of heat stress relative to plants having wild type genotype. In specific embodiments, the polynucleotide molecules of the subject invention encode maize endosperm ADP glucose pyrophosphorylase (AGP) and soluble starch synthase (SSS) enzyme activities. The mutant enzymes confer increased stability of seeds to heat stress conditions during seed development as compared with wild type enzyme activities.

In one embodiment, a mutant polynucleotide of the present invention encodes a large subunit of AGP containing a histidine-to-tyrosine amino acid substitution in the sequence of the polypeptide. This substitution occurs at amino acid residue number 333, according to the accepted number of the amino acids in this protein (Shaw and Hannah, 1992, *supra*). The position of this substitution can be readily identified by a person skilled in the art. A second mutation exemplified in the subject invention is a threonine-to-isoleucine substitution at position number 460 of the AGP protein. Additional mutants confering increased heat stability are shown below in Table 1.

Table 1.

Mutant	Amino Acid Change
HS 13	Ala to Pro at position 177
HS 14	Asp to His at position 400, and Val to Ile at position 454
HS 16	Arg to Thr at position 104
HS 33	His to Tyr at position 333
HS 39	His to Tyr at position 333
HS 40	His to Tyr at position 333, and Thr to Ile at position 460
HS 47	Arg to Pro at position 216, and His to Tyr at position 333

cDNA clones for the subunits of the maize endosperm AGP (SH2 and BT2) and an *E. coli* strain deficient in the endogenous bacterial AGP (*glg* C$^-$) (AC70R1-504) have facilitated the establishment of a bacterial expression system to study the maize endosperm AGP. Expression of a single subunit is unable to complement the *glg* C$^-$ mutant, and no glycogen is produced (Iglesias, A., Barry, G. F., Meyer, C., Bloksberg, L., Nakata, P., Greene, T., Laughlin, M. J., Okita, T. W., Kishore, G. M., and Preiss, J. [1993] *J. Biol Chem.* 268: 1081-86). However, expression of both the large and small subunits on compatible expression vectors fully complements the *glg* C$^-$ mutation and restores glycogen production as evidenced by a dark, reddish-brown staining of colonies exposed to iodine. Thus, complementation is easily identified by simply exposing the colonies to iodine.

In one embodiment, *E. coli glg* C$^-$ cells expressing the structural genes for either potato or maize endosperm AGP were used. Cells containing potato AGP genes can synthesize copious levels of glycogen when grown at 37° or at 42°C. However, cells expressing maize endosperm AGP only synthesize glycogen at 37°C. This result demonstrates the heat sensitivity of wild-type maize endosperm AGP. That there is a difference between potato and maize AGP's in this regard provides an efficient system for screening for mutant cells that have heat stable variants of the maize endosperm AGP.

One aspect of the subject invention pertains to the efficient identification of AGP which is heat stable. Accordingly, a plasmid comprising a polynucleotide encoding for the SH2 subunit of maize AGP was chemically mutagenized, as described below, placed into mutant *E. coli* cells expressing the BT2 subunit, and grown at 42°C. Other mutagens known in the art can also be used. Eleven heritable, iodine staining mutants, termed heat stable (HS) mutants, were isolated. Crude extracts of these mutants were prepared and the heat stability of the resulting AGP was monitored. The mutants retain between 8-59% of their activity after incubation at 60°C for five minutes (Figure 1). This compares to the 1-4% routinely observed for wild-type AGP at this temperature.

The results show that heat stable forms of enzymes can be created according to the subject invention by mutation. Unexpectedly, total activity of the maize endosperm AGP before heat treatment was elevated about 10-fold in the majority of these mutants. This surprising result makes these mutants particularly advantageous for use in agriculture. Mutagenesis techniques as described herein can be used according to the subject invention to identify other genes encoding heat stable starch biosynthesis enzymes.

The genes encoding several of the heat stable mutants, including the two most heat stable HS mutants, HS 33 and HS 40, were completely sequenced. HS 33, which retains 59% of its activity after heat treatment, contains a single base pair mutation that changes a histidine residue at position 333 of the amino acid sequence of the polypeptide to a tyrosine (Figure 2). Primary sequence alignments with the large subunits from wheat and barley AGPs show that a histidine is also present at the analogous residue (Figure 3) (Ainsworth, C., Hosein, F., Tarvis, M., Weir, F., Burrell, M., Devos, K.M., Gale, M.D. [1995] *Planta* 197:1-10). Sequence analysis of HS 40, which retains 41% of its activity post heat treatment, also contained a histidine to tyrosine mutation at position 333. An additional point mutation was identified that generated a threonine to isoleucine substitution. The threonine residue is highly conserved in AGP large subunits, while in AGP small subunits the analogous residue is either a cysteine or serine (Ainsworth *et al.*, 1995, *supra*). The threonine to isoleucine substitution is located close to the carboxyl terminus of the large subunit, and close to a known binding site for the activator 3-PGA (Figure 3).

The subject invention also concerns heat stable mutants of AGP that have mutations in the small subunit of the enzyme. Also encompassed within the scope of the invention are polynucleotides that encode the mutant small subunits of AGP. Mutations in the small subunit of AGP that confer heat stability to the enzyme can also be readily prepared and identified using the methods of the subject invention.

Plants and plant tissue bred to contain or transformed with the mutant polynucleotides, and expressing the polypeptides encoded by the polynucleotides, are also contemplated by the present invention. Plants and plant tissue expressing the mutant polynucleotides produce tissues that have, for example, lower heat-induced loss in weight or yield when subjected to heat stress during development.

The subject invention also concerns methods for producing and identifying polynucleotides and polypeptides contemplated within the scope of the invention. In one embodiment, gene mutation, followed by selection using a bacterial expression system, can be used to isolate polynucleotide molecules that encode enzymes that can alleviate heat-induced loss in starch synthesis in plants.

The subject invention further concerns plants and plant tissue that have an AGP mutant gene incorporated into its genome. Other alleles disclosed herein can also be incorporated into a plant genome. In a preferred embodiment, the plant is a cereal plant. More preferably, the plant is *Zea mays*. Plants having an AGP mutant gene can be grown from seeds that comprise a mutant gene in their genome. In addition, techniques for transforming plants with a gene are known in the art.

Because of the degeneracy of the genetic code, a variety of different polynucleotide sequences can encode each of the variant AGP polypeptides disclosed herein. In addition, it is well within the skill of a person trained in the art to create alternative polynucleotide sequences encoding the same, or essentially the same, polypeptide of the subject invention. These variant or alternative polynucleotide sequences are within the scope of the subject invention. As used herein, references to "essentially the same" sequence refers to sequences which encode amino acid substitutions, deletions, additions, or insertions which do not materially alter the functional activity of the polypeptide encoded by the AGP mutant polypeptide described herein.

Substitution of amino acids other than those specifically exemplified in the mutants disclosed herein are also contemplated within the scope of the present invention. Amino acids can be placed in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby a mutant AGP polypeptide having an amino acid of one class is replaced with another amino acid of the same class fall within the scope of the subject invention so long as the mutant AGP polypeptide having the substitution still retains increased heat stability relative to a wild type polypeptide. Table 2 below provides a listing of examples of amino acids belonging to each class.

Table 2.

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

For example, substitution of the tyrosine at position 333 in the HS 33, HS 39, HS 40 and HS 47 mutant maize endosperm AGP with other amino acids, such as Glycine, Serine, Threonine, Cysteine, Asparagine, and Glutamine, are encompassed within the scope of the invention.

The subject invention also concerns polynucleotides which encode fragments of the full length mutant polypeptide, so long as those fragments retain substantially the same functional activity as full length polypeptide. The fragments of mutant AGP polypeptide encoded by these polynucleotides are also within the scope of the present invention.

The subject invention also contemplates those polynucleotide molecules having sequences which are sufficiently homologous with the wild type *Sh2* DNA sequence so as to permit hybridization with that sequence under standard high-stringency conditions. Such hybridization conditions are conventional in the art (see, *e.g.*, Maniatis, T., E.F.

Fritsch, J. Sambrook [1989] *Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

The polynucleotide molecules of the subject invention can be used to transform plants to express the mutant heat stable AGP enzyme in those plants. In addition, the polynucleotides of the subject invention can be used to express the recombinant variant AGP enzyme. They can also be used as a probe to detect related enzymes. The polynucleotides can also be used as DNA sizing standards.

The polynucleotide molecules of the subject invention also include those polynucleotides that encode AGP enzymes that confer increased seed weight, in addition to enhanced heat stability, on a plant. Combination of a heat stabilizing mutation, HS, with a mutation conferring increased seed weight, *e.g.*, Rev 6, is specifically contemplated for the present invention. See, for example, U.S. Patent Nos. 5,589,618 and 5,650,557.

Mutations in the AGP subunits that confer heat stability can be combined according to the subject invention with phosphate insensitive mutants of maize, such as the *Rev6* mutation, to enhance the stability of the *Rev6* encoded large subunit.

It is expected that enzymic activity of SSS will be impaired at the higher temperature as found with AGP. Thus, mutagenized forms of SSS can be expressed under increased thermal conditions (42°C), to isolate heat stable variants. These heat stable mutagenized forms of SSS are further aspects of the subject invention.

All publications and patents cited herein are hereby incorporated by reference.

Following are examples which illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 – Use of Mutagenesis to Obtain Maize Endosperm AGP Heat Stable Variants

The chemical mutagen hydroxylamine-HCl was initially used for the random mutagenesis of the large subunit expression plasmid. Hydroxylamine preferentially hydroxylates the amino nitrogen at the C-4 position of cytosine, and leads to a GC to AT transition (Suzuki, D.T., Griffith, A.J.F., Miller, J.H., and Lewontin, R.C. [1989] In *Introduction to genetic analysis*, Freeman, NY, 4th ed., pp.475-499). The chemical

mutagen was chosen for its high mutation frequency. Limitations of the chemical mutagen are recognized, and if a large variety of genetic variants are not isolated, PCR based random mutagenesis can be performed. PCR mutagenesis generates a broader spectrum of mutations that include similar frequencies of transitions and transversion, and provides an excellent alternative to the chemical method. The method outlined by Cadwell and Joyce (Cadwell, R.C. and Joyce, G.F. [1992] *PCR Methods and Applications* 2:28-33) can be followed for the PCR based method.

Since the complete expression plasmid is used in the random mutagenesis, it is possible that mutations will occur outside of the coding region. Although it is expected that such mutations will not have any effect on the heat stability of the maize endosperm AGP, each variant can be subcloned into an unmutated expression plasmid before any additional characterization at the enzyme level is conducted. Both the large and small subunit expression plasmids can be constructed so that a *NcoI*/*SacI* digestion will liberate the complete coding region. This can easily be cloned back into a unmutated *NcoI*/*SacI* digested expression plasmid.

Example 2 – Molecular Characterization and Analysis of Heat Stable AGP Variants

Initially, 11 heat stable variants of the maize endosperm large subunit were obtained. Two have been completely sequenced. Sequencing is done using DuPont and ABI instrumentation.

Sequence data can be routinely compared to the progenitor wild-type allele. This analysis reveals the extent of diversity of changes conditioning heat stability.

Both sequenced HS mutants contain the identical histidine to tyrosine change in the large subunit. PCR-derived HS mutants can be quickly screened for the histidine to tyrosine alteration by use of site-specific mutagenesis using primers that change the tyrosine back to histidine.

Example 3 – Expression, Purification, and Kinetic Analysis of Genetic Variants

Conditions for the expression of the wild-type maize endosperm AGP in *E. coli* have been fully characterized. Optimum growth and induction conditions vary somewhat from those previously published for potato AGP expressed in *E. coli* (Iglesias *et al.*,

1993, *supra*; Ballicora *et al.*, 1995, *supra*). Induction at room temperature for 12-14 hrs in the presence of 0.3 mM IPTG and 25 μg/ml nalidixic acid consistently gives high levels of expression and activity. Addition of 30% ammonium sulfate and 10 mM $KH_2PO_4^-/K_2HPO_4^-$ to the extraction buffer stabilizes the maize AGP in the crude extract.

Ammonium sulfate concentrated AGP is further purified by Hydrophobic Interaction Chromatography using Tentacle C3 aminopropyl media (EM Separations) packed into a Pharmacia HR 10/10 column. Protein binds to the column in a buffer containing 1 M ammonium sulfate. AGP is eluted from the column by successive step gradient washes of buffer that contains 0.75 M, 0.5 M, 0.25 M, and 0 M ammonium sulfate. Wild-type maize endosperm AGP typically elutes in the 0.25 M wash. C3 purified maize endosperm AGP is further purified by anion exchange chromatography using Macro-Prep DEAE (BioRad) anion exchange media packed into a Pharmacia HR 10/10 column. AGP is eluted by a linear gradient of 100-500 mM KCl, and typically elutes at a salt concentration around 300 mM. A Pharmacia FPLC system is used for all chromatography steps. The conditions for the individual purification steps are fully characterized. AGP activity during the purification is monitored by the pyrophosphorylysis assay, and purification steps are monitored by SDS-PAGE, Coomassie staining, and Western analysis using polyclonal antibodies specific to the maize endosperm AGP large and small subunits.

Example 4 – Enhanced Subunit Interaction

A totally unexpected pleiotropic effect of the HS maize endosperm AGP mutants is a 2-fold elevation of activity before heat treatment. One possible explanation for this result is that we have, by mutational change, shifted the ratio of SH2 and BT2 monomers and polymers existing within the *E. coli* cell. Perhaps, in wild-type, only 10% or less of the total proteins exist in the active heterotetrameric form whereas in the mutants, this percentage is much higher. If the polymer is more heat resistant than are the monomers, then the phenotype of the mutants would be identical to what has been observed. Kinetic analysis can be used to determine changes in affinities for substrates and/or allosteric effectors.

To test the idea that the monomer/polymer ratio may be altered in these mutants, the amounts of monomers and polymers in wild-type and in selected mutants both before and after heat treatment can be monitored. The availability of antibodies (Giroux, M.J., and Hannah, L.C. [1994] *Mol. Gen. Genetics* 243:400-408) for both subunits makes this approach feasible. This can be examined both through sucrose gradient ultracentrifugation and through gel chromatography and will readily determine which method is most efficient and definitive.

Since the higher plant AGP consists of two similar but distinct subunits that oligomerize to form the native heterotetrameric structure, mutations that enhance this interaction can provide added stability to the enzyme. A yeast two-hybrid system (CLONTECH Laboratories, Palo Alto, CA) can be used to evaluate subunit interactions. Specific primers for the amplification of the coding regions can be constructed. These primers add unique restriction sites to the 5′- and 3′- ends so that cloning facilitates the translational fusion of the individual subunit to the GAL4 DNA binding domain (pGBT9) or GAL4 activation domain (pGAD424). If the proteins cloned into the vectors interact, the DNA binding domain and the activation domain will form a functional transcription activator. This in turn activates expression of the reporter gene, lac Z, cloned behind a GAL4 promoter.

Initially, conditions can be characterized with the wild-type subunits. The coding regions of the wild-type large and small subunits can be cloned into the pGBT9 and pGAD424 yeast expression vectors. All possible combinations can be generated and tested. pGBT9 and pGAD424 vectors containing *Sh2* and *Bt2* can be cotransformed into the same yeast strain, and selected for growth on media lacking tryptophan (pGBT9) and leucine (pGAD424). Subunit interaction as a function of lacZ expression can be detected two ways. Positive colonies are visually identified by a B-galactosidase filter assay. With this assay colonies are bound to the filter, lysed, and incubated with an X-gal solution. Colonies that exhibit a blue color can be analyzed. Subunit interaction can be further analyzed by an enzyme assay specific for B-galactosidase. This allows the quantification of the interaction. Mutations that enhance subunit interactions will give higher levels of B-galactosidase activity when assayed.

Example 5 – Further Enhancement of Stability

The large subunit mutants isolated vary in their heat stability characteristics, suggesting the possibility of multiple mutations. While sequence analysis of mutants HS 33 and HS 40 reveal that the mutant sequences are not identical, both mutants contained the identical histidine to tyrosine change. Given the identification of different HS alterations within the SH2 protein, it is possible to efficiently pyramid these changes into one protein. Furthermore, any HS mutations within the small subunit can be co-expressed with HS SH2 mutants to further enhance the stability of the maize endosperm enzyme.

Multiple HS mutants within one subunit can easily be combined. For example, different unique restriction sites that divide the coding regions of *Sh2* into three distinct fragments can be used. Where appropriate, mutation combinations can be generated by subcloning the corresponding fragment containing the added mutation. If two mutations are in close proximity, then site-directed mutagenesis can be used to engineer such combinations. One method for site specific mutations involves PCR, mutagenic primer, and the use of *Dpn*I restriction endonuclease. Primers can be constructed to contain the mutation in the 5′ end, and used to PCR amplify using the proofreading polymerase Vent. Amplified DNA can then be digested with *Dpn*I. Parental DNA isolated from *E. coli* is methylated and hence susceptible to *Dpn*I. Digested DNA is size fractionated by gel electrophoresis, ligated, and cloned into the expression vectors. Mutations are confirmed by sequence analysis and transformed into the AC70R1-504 strain carrying the wild-type small subunit. Combinatorial mutants can then be analyzed.

Example 6 – Combination of Heat Stability Mutations with *Rev6*

According to the subject invention, the heat stable mutations can be combined with a mutation associated with increased seed weight, such as, for example, the *Rev6* mutation. The goal is to maintain the desired phosphate insensitivity characteristic of *Rev6* while enhancing its stability. Rev 6/HS double mutants can be constructed and confirmed as described herein. Double mutants can be transformed into AC70R1-504 carrying the wild-type small subunit. Increased heat stability can be easily identified by a positive glycogen staining on a low glucose media. *Rev6* does not stain when grown

on this media. Initially all mutant combinations can be screened enzymatically for maintenance of phosphate insensitivity, and only combinations that maintain phosphate insensitivity are further analyzed.

Example 7 – Cloning of SSS I Mutants

A *glg* A$^-$ *E. coli* strain deficient in the endogenous bacterial glycogen synthase can be obtained from the *E. coli* Stock Center. Bacterial expression vectors currently used for the expression of AGP can be used for expression of SSS.

One cloning strategy, as used, for example, with *Sh2* and *Bt2* (Giroux *et al.*, 1996, *supra*), is the following: One primer contains a unique restriction plus the 5′ terminus of the transcript while the other primer contains another unique restriction site and sequences 3′ to the translational termination codon of the gene under investigation. Subsequent cloning of these gives rise to a translational fusion within the plasmid. These gene specific primers are initially used in RT-PCR reactions using poly A+RNA from developing endosperms.

Expression of the maize endosperm SSS I will complement the lack of glycogen synthase activity in the *glg* A$^-$ strain. Complementation should be easily visualized with iodine staining as it is with the expression of AGP in the *glg* C$^-$ strain. Crude extracts can be incubated at various temperatures and lengths of time to determine the heat stability of SSS I. The *glg* A$^-$ strain expressing the maize endosperm SSS I can be grown at various temperatures to determine if function is temperature sensitive as it is with the AGP bacterial expression system. Once a restrictive temperature is established, a random mutagenesis can be conducted with the SSS I clone. Mutant forms of SSS I can be transformed into the *glg* A$^-$ strain, grown at the restrictive temperature, and heat stable variants identified by their ability to produce iodine-staining glycogen at the restriction temperature.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

Claims

We claim:

1. A polynucleotide, or a fragment or variant thereof, encoding a mutant AGP polypeptide, wherein said mutant AGP polypeptide exhibits increased heat stability relative to a wild type AGP polypeptide.

2. The polynucleotide according to claim 1, wherein said mutant polypeptide encoded by said polynucleotide is a plant AGP polypeptide.

3. The polynucleotide according to claim 1, wherein said mutant polypeptide encoded by said polynucleotide comprises an amino acid mutation in the large subunit of said polypeptide.

4. The polynucleotide according to claim 1, wherein said mutant polypeptide encoded by said polynucleotide comprises an amino acid mutation in the small subunit.

5. The polynucleotide according to claim 3, wherein said mutant polypeptide encoded by said polynucleotide comprises an amino acid mutation wherein a histidine residue at position 333 in the amino acid sequence of said polypeptide is replaced by an amino acid that confers heat stability to said polypeptide.

6. The polynucleotide according to claim 5, wherein said amino acid substituted for histidine at position residue number 333 is a tyrosine.

7. The polynucleotide according to claim 3, wherein said mutant polypeptide encoded by said polynucleotide comprises an amino acid mutation wherein an alanine residue at position 177 in the amino acid sequence of said polypeptide is replaced by an amino acid that confers heat stability to said polypeptide.

8. The polynucleotide according to claim 7, wherein said amino acid substituted for alanine at position residue number 177 is a proline.

9. The polynucleotide according to claim 3, wherein said mutant polypeptide encoded by said polynucleotide comprises an amino acid mutation wherein an aspartic acid residue at position 400 in the amino acid sequence of said polypeptide is replaced by an amino acid that confers heat stability to said polypeptide.

10. The polynucleotide according to claim 9, wherein said amino acid substituted for aspartic acid at position residue number 400 is a histidine.

11. The polynucleotide according to claim 3, wherein said mutant polypeptide encoded by said polynucleotide comprises an amino acid mutation wherein a valine residue at position 454 in the amino acid sequence of said polypeptide is replaced by an amino acid that confers heat stability to said polypeptide.

12. The polynucleotide according to claim 11, wherein said amino acid substituted for valine at position residue number 454 is an isoleucine.

13. The polynucleotide according to claim 3, wherein said mutant polypeptide encoded by said polynucleotide comprises an amino acid mutation wherein an arginine residue at position 104 in the amino acid sequence of said polypeptide is replaced by an amino acid that confers heat stability to said polypeptide.

14. The polynucleotide according to claim 13, wherein said amino acid substituted for arginine at position residue number 104 is a threonine.

15. The polynucleotide according to claim 3, wherein said mutant polypeptide encoded by said polynucleotide comprises an amino acid mutation wherein a threonine residue at position 460 in the amino acid sequence of said polypeptide is replaced by an amino acid that confers heat stability to said polypeptide.

16. The polynucleotide according to claim 15, wherein said amino acid substituted for threonine at position residue number 460 is an isoleucine.

17. The polynucleotide according to claim 3, wherein said mutant polypeptide encoded by said polynucleotide comprises an amino acid mutation wherein an arginine residue at position 216 in the amino acid sequence of said polypeptide is replaced by an amino acid that confers heat stability to said polypeptide.

18. The polynucleotide according to claim 17, wherein said amino acid substituted for arginine at position residue number 216 is a proline.

19. A method for increasing heat resistance of a plant, said method comprising incorporating the polynucleotide of claim 1 into the genome of said plant and expressing the protein encoded by said polynucleotide molecule.

20. The method according to claim 19, wherein said plant is *Zea mays*.

21. A plant expressing the polynucleotide molecule of claim 1.

22. The plant according to claim 21, wherein said plant is *Zea mays*.

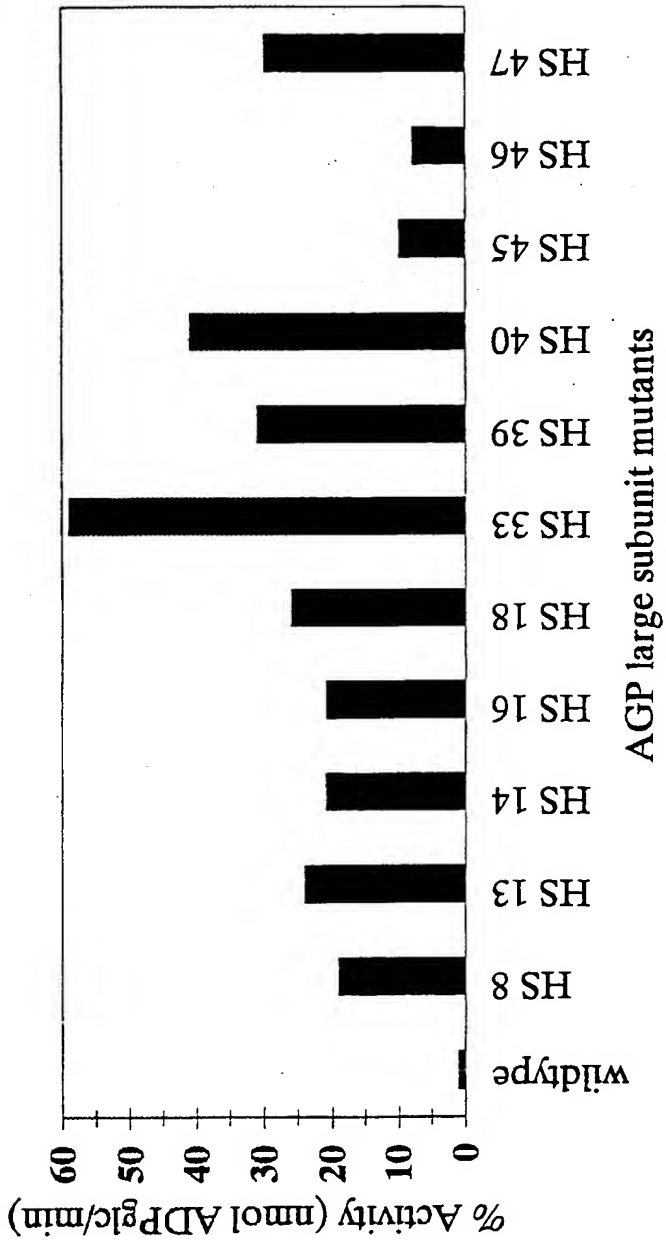
23. A plant tissue comprising the polynucleotide molecule of claim 1 within the genome of said tissue.

24. The plant tissue according to claim 23, wherein said plant seed is produced by *Zea mays*.

25. The plant tissue according to claim 23, wherein said plant tissue is a seed.

26. A mutant AGP polypeptide encoded by the polynucleotide of claim 1.

27. The polynucleotide according to claim 1, wherein said mutant polypeptide encoded by said polynucleotide further comprises an amino acid mutation that confers increased seed weight to a plant expressing said polynucleotide.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10
20
30
40
50
60
wildtype
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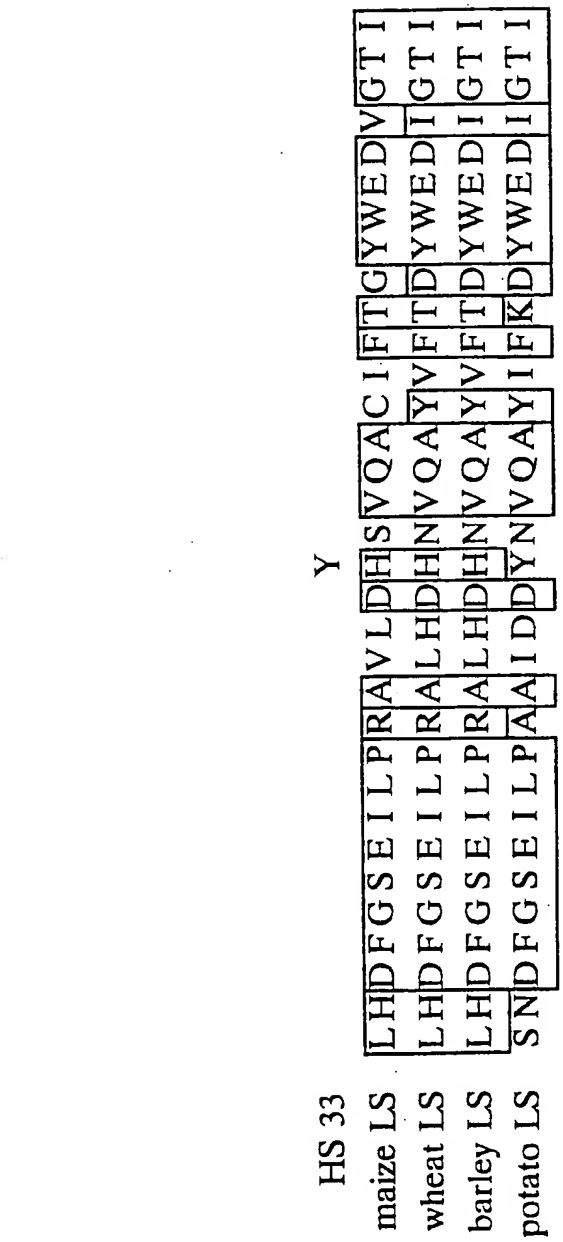
HS 14
HS 16
HS 18
HS 33
HS 39
HS 40
HS 45
HS 46
HS 47
AGP large subunit mutants

Figure 1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maize LS
wheat LS
barley LS
potato LS
Y
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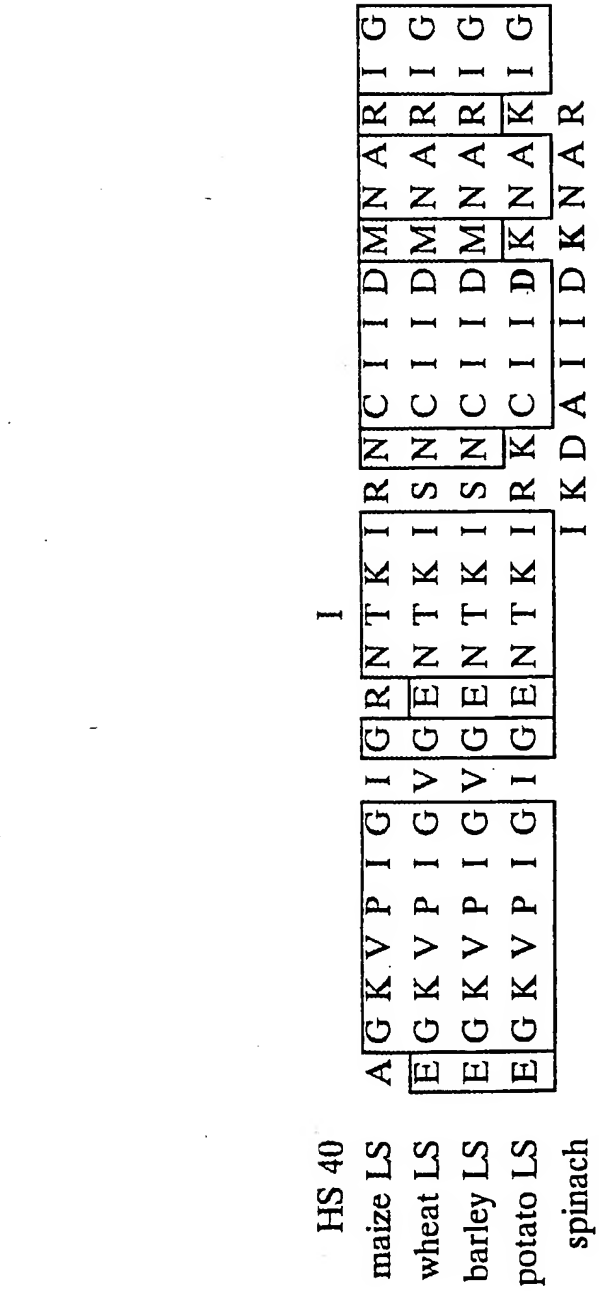
Figure 2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wheat LS E G K V P I G V G E N T K I S N C I I D M N A R I G
barley LS E G K V P I G V G E N T K I S N C I I D M N A R I G
potato LS E G K V P I G I G E N T K I R K C I I D K N A K I G
spinach LS I K D A I I D K N A R

Figure 3

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 97/21145

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N15/54 C12N9/12 C12Q1/68 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12Q A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	WO 94 09144 A (ZENECA LTD) 28 April 1994 pages 1-5, 7,8,9	1-27

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 March 1998	08/04/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Holtorf, S

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INTERNATIONAL SEARCH REPORT

Inter ional Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	WO 95 34660 A (CAMBRIDGE ADVANCED TECH ;BURRELL MICHAEL MEYRICK (GB); COATES STEP) 21 December 1995 see the whole document ---	1-27
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